Title: Establishment and Functions of DNA Methylation in the Germline

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Abstract

Epigenetic modifications established during gametogenesis regulate transcription and other nuclear processes in gametes, but also have influences in the zygote, embryo and postnatal life. This is best understood for DNA methylation which, established at discrete regions of the oocyte and sperm genomes, governs genomic imprinting. In this review, we describe how imprinting has informed our understanding of *de novo* DNA methylation mechanisms, highlight how recent genome-wide profiling studies have provided unprecedented insights into establishment of the sperm and oocyte methylomes, and consider the fate and function of gametic methylation and other epigenetic modifications after fertilisation.

Introduction

Epigenetic marks are a fundamental component of the mechanisms that functionally interpret DNA sequence. These marks contribute to the establishment and maintenance of specialised gene expression patterns that determine cell identity. Epigenetically reinforced transcriptional states can be propagated through cell division, acting as a long-term marker of developmental origin during lineage specification. DNA methylation is an epigenetic mark that can be established *de novo*, maintained through cell division, and be interpreted by transcription machinery and DNA-binding proteins. It is a repressive mark that characterises heterochromatin in mammalian cells, and when found at gene promoters DNA methylation generally suppresses transcription [1].

DNA methylation involves the transfer of a methyl group from S-adenosyl-l-methionine to a cytosine residue [2]. The process is catalysed by DNA methyltransferases (DNMTs) in two distinct modes. During cell division, replication of a methylated DNA sequence creates hemimethylated CpG dinucleotides, where methylation is found only on the original strand and is absent from the newly synthesised strand. Hemimethylated CpGs attract the maintenance methyltransferase DNMT1, which methylates the unmethylated strand to restore symmetric DNA methylation [3-6]. In contrast, the *de novo* methyltransferases DNMT3A and DNMT3B methylate CpGs unmethylated on both DNA strands [7]. The related protein DNMT3L lacks a catalytic domain but acts as a crucial cofactor of both DNMT3A and DNMT3B [8-12].

DNA methylation establishment in the germline is of particular importance, as methylation marks established in the gametes have the potential to regulate gene expression in the next generation. Imprinted loci are the paradigm for this inter-generational mode of gene regulation. Failure to establish correct germline-specific DNA methylation patterns has serious consequences for post-fertilisation development. However, the mechanisms that target *de novo* methylation in the

germline are not fully understood. In this review, we will highlight recent advances in elucidating the mechanisms that direct *de novo* methylation in the germline, particularly in the female germline, and will review current knowledge of the roles of gametic DNA methylation in gametogenesis and post-fertilisation development.

DNA methylation in genomic imprinting

Initially, DNA methylation in gametes was studied largely from the point of view of imprinted germline differentially methylated regions, or imprinted gDMRs. Imprinted gDMRs are loci that are methylated in either the female (maternally imprinted) or the male (paternally imprinted) germline, and that retain this monoallelic methylation in a parent-of-origin specific manner following fertilisation [13]. In somatic cells, the maternal and paternal alleles of imprinted loci have different epigenetic landscapes, composed of distinct DNA methylation profiles, histone post-translational modifications, and regulation by non-coding RNAs. Human and mouse are the two most extensively used models for imprinting studies, although genomic imprinting is also found and well-characterized in flowering plants [14]. Imprinted loci represent a unique mode of epigenetic regulation, and a number of human congenital disorders are caused by loss of imprinting, making genomic imprinting a valuable model for both basic and translational research questions [15].

In mouse, there are twenty-three definitive and eleven putative maternally imprinted gDMRs [16] and three paternally imprinted gDMRs [13]. Maternally imprinted gDMRs fall within a category of CpG-rich genomic regions called CpG islands (CGIs), while the paternal gDMRs have CpG densities closer to the genome average [17, 18] (Figure 1). The vast majority of CpG dinucleotides in most cell types are methylated, but the CpGs that comprise CGIs are exceptions to this rule and remain unmethylated in most cellular contexts. Functionally, CGIs are found at the majority of mammalian gene promoters, and their resistance to DNA methylation allows the RNA polymerase complex and relevant transcription factors to access the transcription start sites of genes [19]. Although CGIs canonically lack DNA methylation, in a typical mammalian cell a small proportion of these loci are fully methylated, often in a tissue-specific manner [20-22]. The CGIs that gain methylation within a particular cell type are most often intragenic, and are found within an actively transcribed gene [23]. The propensity for a CGI to gain methylation is also influenced by its CpG density, GC content, and enrichment for transcription factor-binding motifs [24-27].

Prior to the advent of genome-wide analyses, the general assumption was that imprinted gDMRs were specifically targeted in the germline by specialised *de novo* methylation machinery, and

research efforts were concentrated on searching for features that distinguished these regions from the rest of the genome. Genome-wide methylation studies have led to a reappraisal of this view. Specifically, comparing the oocyte and sperm methylomes revealed the existence of many more regions of differential methylation between the gametes than the classic imprinted gDMRs [28, 29]. We will refer to these additional regions as non-imprinted gDMRs. In addition to the maternally imprinted gDMRs, the oocyte methylome contains many additional methylated but non-imprinted CGIs, which have a similar sequence composition to the imprinted CGIs. Similarly, the paternally imprinted gDMRs do not seem to be distinct from many of the other genomic regions that are methylated in sperm [28, 29]. Therefore, rather than being uniquely targeted for DNA methylation, it is now thought that imprinted gDMRs gain methylation in the same manner as analogous regions in the genome (Figure 1). The distinguishing feature of imprinted gDMRs compared to non-imprinted gDMRs, then, is that the differential methylation at imprinted gDMRs persists following fertilisation and epigenetic reprogramming in the early embryo, while the differential methylation at non-imprinted gDMRs does not [13]. What remains unclear is exactly how the differential patterns of DNA methylation that give rise to imprinted and non-imprinted gDMRs are established in sperm and oocytes.

DNA methylation establishment in gametes

Timing of DNA methylation establishment

At the time of sex determination in the fetal gonads, which in mice occurs at around embryonic day 12.5 (E12.5), global DNA methylation levels are very low, and the methylomes of male and female primordial germ cells (PGCs) are very similar [30, 31]. However, *de novo* methylation establishment proceeds in very different manners in the male and female germlines. In the male germline, *de novo* methylation is initiated at embryonic day E13.5, in prospermatogonia or gonocytes that are arrested in mitosis, and the male methylome is completely established prior to birth [31-33] (Figure 2). Following methylation establishment, male gametes experience a proliferative burst before entering meiosis and beginning the process of differentiating into sperm. Therefore, the mature sperm methylome is a product of DNA methylation maintenance that must operate faithfully from birth in male mice.

In contrast, *de novo* methylation is not initiated until after birth in the female germline. In oocytes, DNA methylation establishment occurs in parallel with the follicular growth phase of oogenesis and is largely complete by the germinal vesicle (GV) stage, at approximately P21 in mice [28]. Several

studies have demonstrated that DNA methylation establishment, at least at imprinted gDMRs, is coordinated with oocyte growth, and loci gain methylation at different stages of growth in a regulated manner [34-36] (Figure 2). The function of and mechanisms underlying this asynchronous DNA methylation establishment in oocytes remains unclear, and whether or not the paternally imprinted gDMRs are similarly methylated in coordination with spermatogenesis progression has not been extensively studied. Oocytes are arrested in meiotic prophase I throughout the growth phase, and do not resume meiosis until ovulation. Therefore, *de novo* methylation occurs on a non-replicating genome in both the male and female germlines, but only the oocyte methylome is solely reflective of *de novo* methylation events.

Mechanisms underlying DNA methylation establishment

Between the male and female germlines, the male provides much more starting material for analysis. Additionally, unlike in oocytes, loss of DNA methylation during spermatogenesis causes infertility accompanied by a profound reduction in testis size, making factors important in DNA methylation establishment in the paternal germline readily identifiable. Methylation establishment during spermatogenesis has therefore been extensively studied using standard molecular and genetic approaches, and the mechanisms underlying *de novo* methylation in male germ cells are well-characterised. DNA methylation establishment in male germ cells is initiated at repetitive sequences by a testis-specific class of small RNAs called PIWI-interacting RNAs, or piRNAs. These piRNAs are derived from repeat sequences and direct methylation and silencing of transposable elements in male germline, as well as of *Rasgfr1*, a paternally imprinted locus that contains a retrotransposon sequence in its gDMR [37]. The interplay between the piRNA pathway and the DNA methyltransferase enzymes remains incompletely understood, however, piRNAs appear to act upstream of DNMT3L, as loss of DNMT3L does not affect piRNA expression, while piRNA deficiency leads to loss of methylation at transposable elements [38, 39].

Before genome-wide studies were possible, the mechanisms underlying DNA methylation in the germlines were poorly understood. However, maternally imprinted gDMRs presented a potentially more interesting context for studying *de novo* methylation, since there are more maternally imprinted than paternally imprinted gDMRs, and maternally imprinted gDMRs are also CGIs, a genomic feature mostly unmethylated in mammalian genomes. Several lines of evidence had suggested that transcription might be a common requirement for methylation establishment at maternally imprinted gDMRs. This was first functionally demonstrated for the complex imprinted locus *Gnas*, where interrupting transcription from an upstream oocyte-specific promoter led to loss

of methylation at the downstream maternally imprinted gDMR [40, 41]. Similar results were subsequently obtained for the gDMRs of the *Snrpn* and *Zac1* imprinted loci [42, 43].

More recently, genome-wide methylation analyses have provided a holistic view of the sperm and oocyte methylomes. Genetic studies have revealed DNA methylation establishment in both oocytes and sperm is primarily due to the activity of DNMT3A and its cofactor DNMT3L [44-46]. DNMT3B is required for methylation at the *Rasgrf1* imprinted gDMR and at satellites in sperm [33], but is dispensable for sperm production, and plays no role in the oocyte methylome [47].

Despite being established by the same DNMT3A/DNMT3L complex, the sperm methylome is very different from the oocyte methylome: around 90% of CpGs are methylated in sperm, compared with 40% in oocytes [29, 48]. Additionally, the distribution of DNA methylation differs greatly between oocytes and sperm: the oocyte methylome is characterized by striking alternating hypermethylated and hypomethylated domains, while in sperm DNA methylation is distributed more or less evenly throughout the genome, with the notable exception of CGIs (Figure 1). In support of a more general role for transcription in *de novo* DNA methylation in oocytes, hypermethylated and hypomethylated domains are respectively correlated with active transcription units, and intergenic or inactive genomic regions [29, 43]. In sperm, both genic and intergenic regions gain methylation [29, 48, 49] (Figure 1). Therefore, it appears that DNA methylation targeting by transcription is a particular feature of the female germline, while methylation is established more indiscriminately in the male germline. Nevertheless, this apparently indiscriminate methylation establishment might also be associated with transcription, as low-level genome-wide transcription has been detected in prospermatogonia preceding DNA methylation establishment, including across paternally-imprinted gDMRs [49]. The functional significance of transcription in patterning the sperm methylome has not yet been tested.

To precisely quantitate the correlation between transcription and DNA methylation in oocytes, we recently comprehensively mapped oocyte transcription units [43]. A number of oocyte-specific transcripts that traversed maternally imprinted gDMRs had already been described, and we therefore expected that the oocyte transcriptome would include many additional oocyte-specific transcripts not found in the mouse reference transcriptome. We identified ~9000 novel transcription units, some with protein-coding potential, and novel upstream promoters for almost 2000 annotated genes. This demonstrates that expression in the oocyte is uniquely regulated, and the functional consequences of this unique regulation may not be limited to DNA methylation establishment. Using the oocyte transcriptome, ~90% of the hypermethylated domains in the oocyte genome were correlated with transcription and, similarly, ~90% of hypomethylated domains were

confirmed as untranscribed [43]. Though exceptions remain, this work demonstrates an exquisite correlation between transcription and DNA methylation in oocytes.

Histone modifications and DNA methylation establishment

DNA methylation correlates with histone modifications

DNA methylation is known to correlate and anti-correlate with a number of different histone modifications. It is perhaps best-studied in relation to histone lysine methylation, a regulatory mark that recruits or repels chromatin-associated proteins via specific reader domains [50]. Through exclusion of the DNA methyltransferase complex, methylated histone H3 lysine 4 (H3K4) protects associated DNA from DNA methylation [51-53]. Both tri- and dimethyl H3K4 are classic marks of active promoters and CGIs [20, 54] and, as a result, these regions usually lack DNA methylation. Histone H3 lysine 36 trimethylation (H3K36me3) is also associated with transcriptionally active chromatin, and is laid down in gene bodies by factors associated with the elongating RNApolII complex [55, 56]. Unlike H3K4me2 and H3K4me3, H3K36me3 positively correlates with DNA methylation, and in most mammalian cells gene bodies are marked by both H3K36me3 and DNA methylation [57]. Additionally, DNA methylation and histone H3 lysine 9 (H3K9) methylation co-occur at heterochromatic regions. The relationship between DNA methylation and histone lysine methylation is well-documented in many cell contexts and has been reviewed elsewhere [58, 59].

Histone modifications can direct *de novo* DNA methylation

In systems that begin with little or no DNA methylation, such as male and female PGCs, the histone modification landscape may influence DNA methylation by directing the DNA methyltransferase machinery [59, 60]. Most work addressing the roles of histone modifications in DNA methylation establishment has been done in systems that have been manipulated to lack DNA methylation, such as mouse embryonic stem cells (mESCs) that lack the methyltransferases DNMT3A, DNMT3B and DNMT1 [61]. This system has been used to interrogate DNMT3A and DNMT3B recruitment to chromatin using rescue experiments that re-introduce one or both methyltransferases. Using such a system, Baubec and colleagues recently demonstrated that H3K36me3-marked regions gain DNA methylation when DNMT3A and, particularly, DNMT3B are re-introduced [62]. When murine DNMT3B is introduced in yeast, which lack endogenous DNA methylation, it similarly methylates H3K36me3-associated DNA [63]. H3K36me3 can recruit DNMT3A via its PWWP domain *in vitro* [64], and in the mouse oocyte DNMT3A is the only active methyltransferase [47] , suggesting that in the absence of DNMT3B it could be recruited to H3K36me3-marked DNA and establish DNA

methylation. Recruitment of the DNMT3A/DNMT3L methyltransferase complex by H3K36me3 could therefore be the mechanism underlying the tight correlation observed between transcription and DNA methylation in oocytes.

Recruitment of DNMT3A and DNMT3B to chromatin has been extensively studied *in vitro*, but the *de novo* DNA methylation that occurs *in vivo* during gametogenesis and early embryogenesis can further inform our understanding of how histone modifications and DNA methylation influence one another. In developing and mature sperm, which have been profiled using both whole-genome bisulfite sequencing and ChIP-Seq methods, H3K4me2- and H3K4me3-marked regions remain unmethylated, while the majority of other CpG dinucleotides gain methylation [48, 49]. This pattern of DNA methylation is seen in somatic cells, but seeing the same result in sperm provides stronger evidence that histone modifications pattern *de novo* DNA methylation, since in prospermatogonia DNA methylation establishment takes place on a largely unmethylated genome. Notably, 99% of histones in mature mouse sperm are exchanged for protamines – small, arginine-rich proteins which allow DNA to condense more than canonical nucleosomes [65] (Figure 2). Retained histones are disproportionately found at CGIs and are characterised by H3K4me2 and H3K4me3, suggesting that histones may be retained at these loci specifically to prevent *de novo* methylation [48, 66, 67].

It is now well-established that H3K4me2 and H3K4me3 protect associated DNA from DNA methylation, and that this inhibition protects most CGIs and transcriptional start sites from aberrantly acquiring DNA methylation [68]. To establish DNA methylation at a CGI, such as a maternally imprinted DMR, H3K4me2 and H3K4me3 therefore would first have to be removed. Single-gene analyses of oocytes from mice deficient in the H3K4me2 demethylase KDM1B have shown that several maternally imprinted gDMRs fail to gain methylation in the absence of this enzyme [69]. This suggests that H3K4me2 is aberrantly retained at the maternally imprinted gDMRs that are unmethylated in the absence of KDM1B. Most CGIs that gain methylation in the oocyte, both imprinted and non-imprinted, are found within active transcription units, suggesting they are enriched for H3K36me3. Unlike sperm, oocytes are difficult to isolate in the numbers required for standard ChIP-Seq, so although whole-genome bisulfite sequencing of oocytes has now been done by several groups, comparing the oocyte methylome to the histone modification landscapes in oocytes was not previously possible.

We recently interrogated the mechanistic links between histone modifications and transcription in oocytes using two different approaches. Disrupting transcription at the imprinted *Zac1* locus through genetic deletion of the oocyte-specific *Zac1o* promoter led to loss of DNA methylation throughout the locus, which was accompanied by loss of H3K36me3 in the *Zac1* gene body and retention of

H3K4me2 at the *Zac1* imprinted gDMR [43]. This demonstrated a functional requirement for transcription in DNA methylation establishment. It also showed that in the absence of transcription CGI chromatin is not remodeled, and the histone modification landscape at a CGI that normally gains DNA methylation in oocytes remains incompatible with DNA methylation establishment. Genome-wide methylation profiling in oocytes lacking KDM1B revealed widespread loss of methylation in gene bodies and imprinted gDMRs, including the *Zac1* gDMR [70]. RNA-seq revealed that transcription is largely normal in KDM1B-deficient oocytes, indicating KDM1B acts downstream of transcription.

Although the majority of measurable CGIs were hypomethylated in KDM1B-knockout oocytes compared to wild-type, a subset of CGIs fully gained methylation in the knockout oocytes, indicating KDM1B is not universally required for CGI methylation. Using novel techniques for oocyte isolation and low-cell ChIP-Seq, we noted that CGIs sensitive to KDM1B had higher H3K4me2 levels in wild-type oocytes than other CGIs, which suggests that the demethylase activity of KDM1B is required at these loci for proper establishment of DNA methylation [70]. To confirm this correlation between higher H3K4me2 levels and a requirement for KDM1B, future experiments should include ChIP-seq in KDM1B-knockout oocytes. We also observed an increase between the primary and growing oocyte stages in H3K36me3 at CGIs destined for DNA methylation (Figure 3), suggesting this is a general mechanism that specifies these loci for subsequent methylation [70].

Recently, Nashun and colleagues reported widespread hypomethylation in oocytes deficient in the H3.3 chaperone HIRA [71]. HIRA deposits H3.3 in a replication-independent manner at euchromatin [72], and in the non-dividing mouse oocyte deleting HIRA caused reduced histone load genome-wide [71]. HIRA-knockout oocytes showed DNA hypomethylation even in regions that were transcribed normally, indicating that the requirement for a specific histone modification profile in *de novo* DNA methylation is subordinate to a general requirement for intact nucleosomes. This makes sense, given structural and biochemical data that show the DNMT3A/DNMT3L complex is activated through interaction with the histone H3 tail [73, 74].

Taken together, these results indicate that in oocytes transcription facilitates establishment of chromatin states that permit DNA methylation, especially at CGIs that are normally marked with inhibitory H3K4 methylation [43, 70]. However, this chromatin remodelling may not be achieved through a single histone modifier, as evidenced by the subset of oocyte-methylated CGIs that gain normal DNA methylation levels in the absence of KDM1B. Although the mature oocyte methylome is highly stereotypical, the histone modification landscape in these methylated domains is not uniform prior to *de novo* methylation [70]. As a consequence of this, it is likely that a different balance of

factors, including different histone modifiers, is required at different genomic locations to render the DNA in these regions susceptible to DNA methylation. For example, CpG-dense CGIs, which have high H3K4me2 and H3K4me3, may require active removal of this mark prior to methylation establishment (Figure 3), while at CpG-poor CGIs deposition of H3K36me3 alone may be sufficient to target DNA methylation.

Unanswered questions in DNA methylation establishment

The link between transcription-associated histone modifications and DNA methyltransferase recruitment can, at least in theory, largely explain the oocyte methylation landscape. However, it also raises the question of why the DNA methyltransferases, specifically DNMT3A and DNMT3B, seem preferentially to target genomic features in a cell type-specific manner. In mESCs, where both DNMT3A and DNMT3B are present, it is DNMT3B, not DNMT3A, that preferentially methylates genic regions [62]. DNMT3A and DNMT3B have virtually identical PWWP domains, which both bind to H3K36me3 [64, 75]. It is therefore unclear why this preferential targeting of DNMT3B would occur when both enzymes are present, as is the case in mESCs. It could be evidence for DNMT3B being more important generally in an embryonic context; DNMT3B knockout mice die mid-gestation, while DNMT3A knockout mice die much later, at around weaning [45]. Also unclear are the mechanisms underlying the very different methylomes seen in mature oocytes and sperm. Why would DNMT3A/3L be so precisely targeted to genic regions in the oocyte, but be so indiscriminate in sperm? Adding another layer of complexity, recent profiling of the human oocyte methylome shows the same genic/intergenic patterning of hypermethylated and hypomethylated domains seen in mouse [76], yet human oocytes lack DNMT3L [77]. This suggests that the oocyte methylome is determined through DNMT3A targeting alone, or that it is a consequence of some as-yet unidentified aspect of oocyte biology.

De novo methylation occurs prior to histone-protamine exchange in spermatogenesis, so in theory post-translational histone modifications could direct methylation in this context, but there is no clear correlation between any such modification and the genome-wide methylation seen in sperm. Instead, the sperm methylome is generally similar to the methylomes of somatic cells, in which >70% of CpG dinucleotides are methylated. But it is important to note that sequence-independent methylation establishment in both the male and female germlines allows for the epigenetic plasticity that underlies sex-specific *de novo* methylation of imprinted gDMRs, and evolutionarily creates the opportunity for new imprinted gDMRs to arise.

Roles of DNA methylation in sperm and oocytes

DNA methylation is essential for the correct development of the male germline. DNMT3A or DNMT3L deficiency leads to meiotic failure, apoptotic loss of spermatocytes and sterility. This is presumably due to the down-regulation of germ cell-specific genes and transcriptional activation of transposable elements that become capable of retrotransposition [46, 78-80]. These phenotypes are accompanied by genome-wide hypomethylation. DNA methylation in the male germline has also recently been shown to play a role in preventing retrotransposons from participating in meiotic recombination [81].

Though the oocyte has many unique epigenetic features, the most perplexing may be its unique methylome. Unlike the methylome of any other differentiated mammalian cell, the oocyte methylome is predominantly genic, with almost no methylation in intergenic regions. This patterning bears a strong resemblance to invertebrate methylomes, and is therefore probably very ancient [82]. Why has the oocyte retained this distribution of CpG methylation, when no other mammalian cell analyzed thus far has? Unfortunately, the functional role of methylation in the oocyte is not very informative in this regard; oocytes can mature, progress through meiosis and become fertilised in the absence of DNA methylation [8, 78, 83]. Thus far, the only definitive roles for oocyte DNA methylation are in post-fertilisation contexts. Although DNMT3A- and DNMT3L-deficient oocytes can be fertilised normally, embryos conceived from these oocytes die by E10.5 [78], largely because of the absence of methylation at maternally imprinted gDMRs.

Apart from imprinted gDMRs, the majority of methylation marks established in gametes are erased after fertilisation. Nevertheless, some regions other than imprinted gDMRs maintain gametic methylation post-fertilisation [28, 29, 84]. In the majority of cases, these regions retain methylation from oocytes on the maternally-inherited allele, while the paternal allele gains methylation during postimplantation development [21, 85-87]. These observations have given rise to the concept of transient DMRs [86]. It remains unclear whether non-imprinted, oocyte-derived methylation has any major function during embryonic development, however, two recent studies revealed that at some loci, this methylation plays a role in transcriptional regulation in the embryo [87, 88]. More recently, maternal methylation has also been shown to be required in extra-embryonic tissue, specifically in trophoblast. Conceptuses from DNMT3A-deficient oocytes, which lack DNA methylation, exhibited misregulated transcription and developmental defects in trophoblast cells [89]. Advances in

genome-wide analyses of rare cells may reveal further roles for maternally-derived methylation in early development.

The oocyte's ability to progress normally through oogenesis in the absence of DNA methylation suggests it employs alternative mechanisms to regulate gene expression and repress repetitive elements. Oocytes lacking the nucleosome remodeler LSH exhibit meiotic defects, hypomethylation of repeat elements, and retrotransposon reactivation, echoing the phenotype seen in DNMT3L-deficient spermatocytes [90]. Throughout oogenesis, oocytes are heavily reliant on extracellular signaling from other cells within the follicle, including to initiate translation of mRNAs related to meiotic progression and developmental competence [91]. This extreme dependence on non cell-autonomous mechanisms of gene regulation may explain the oocyte's ability to tolerate a lack of DNA methylation, and represents a unique system of transcriptional regulation among mammalian cells.

Fate of gametic DNA methylation after fertilisation

The maternal and paternal pronuclei are differentially marked with both DNA methylation and histone modifications in the early zygote. The paternal pronucleus is largely devoid of histones and is heavily DNA-methylated, while the maternal pronucleus is organised around canonical histones bearing post-translational modifications and is DNA-methylated primarily in genic regions. After fertilisation, the paternal pronucleus is extensively remodeled in a process that entails active DNA demethylation [92, 93] and protamine-histone exchange characterised by deposition of maternal H3.3 [94, 95] (Figure 4). The maternal pronucleus largely resists this initial wave of demethylation, although it has recently been shown that the maternal genome is subject to both *de novo* methylation and hydroxylation in the zygote [96]. Instead, through failure to maintain DNA methylation following DNA replication, the maternal genome passively loses methylation in successive cell divisions. Following implantation, DNA methylation is re-established in the embryonic and extra-embryonic lineages, through processes that have been reviewed elsewhere [97].

Gametic methylation and transgenerational Inheritance

There has been much speculation about the possibility of transgenerational epigenetic inheritance in mammals [98]. Studies of transgenerational epigenetic inheritance mostly focus on paternal inheritance, as this avoids confounding *in utero* effects and other maternal influences that

complicate studies of maternal inheritance. It has been shown by multiple groups that the sperm methylome can be perturbed by environmental influences [99-101]. However, Shea and colleagues demonstrated recently that sibling mice that were fed different diets had more similar sperm methylomes than non-siblings on identical diets, demonstrating that naturally-occurring epigenetic variation influences the sperm methylome more than paternal diet [102]. DNA methylation changes in sperm may therefore not have a direct effect on offspring.

It is thought that diffusible factors from the gametes, such as RNAs, may in general be a greater source of transgenerational epigenetic information than DNA methylation [103]. A recent study demonstrating that tRNA composition in sperm is affected by paternal diet, and that these tRNA fragments can regulate genes that are highly expressed in the pre-implantation embryo, provides an alternative plausible mechanism for paternal transgenerational influence [104]. Nonetheless, some genomic loci, in particular intracisternal A particles (IAPs), fail to become completely demethylated prior to *de novo* methylation in PGCs, providing a potential source of transgenerational information via DNA methylation [31, 105], and the consequences of such retained methylation remain to be uncovered.

Histone modifications underlie asymmetric DNA methylation in the zygote

The gametes do not only contribute their unique methylomes to the zygote; the epigenetic information contained in modified histones is also transmitted at fertilisation. Sperm, oocytes, zygotes and the early embryo have been extensively studied using immunofluorescence and genetic methods, which have provided insights into the histone modifications that characterise these cells. Several lines of evidence suggest that, as in DNA methylation establishment, histone modifications may influence DNA methylation retention and loss in the zygote (Figure 4).

De novo DNA methylation is directed by the distributions of active histone marks in the gametes: H3K4me2 and H3K4me3 in sperm; and H3K4me2, H3K4me3, and H3K36me3 in oocytes. DNA methylation retention in the zygote, conversely, is reliant on co-occurrence of DNA methylation and heterochromatic marks: H3K9me2, H3K9me3, and histone H3 lysine 27 trimethylation (H3K27me3). PGC7/STELLA is a maternal factor that binds H3K9me2 and is required to protect the maternal pronucleus from DNA demethylation [106, 107] (Figure 4). Reducing H3K9me2 levels causes loss of PGC7/STELLA binding and loss of methylation in the maternal pronucleus, indicating that in wild-type zygotes it is the absence of H3K9me2 that renders the paternal genome vulnerable to DNA demethylation. Further, two of the three paternally imprinted gDMRs are marked with H3K9me2 in

sperm and are hypomethylated in PCG7-knockout embryos, suggesting the PCG7/STELLA interaction with H3K9me2 is a general mechanism for DNA methylation retention in the zygote [107]. Polycomb Group factors are specifically required to maintain imprinted gene expression in the embryo, suggesting H3K27me3, which is deposited by the Polycomb Group component EZH2, is also present on imprinted gDMRs in the zygote [108, 109].

DNA methylation and histone modifications in the early embryo

Both maternally and paternally imprinted gDMRs retain monoallelic methylation in the zygote, early embryo, and post-implantation [13]. Maternally-derived, non-imprinted methylation is mostly removed by the morula stage but, intriguingly, the extra-embryonic lineages are characterised by a gene body methylation signature reminiscent of the oocyte methylome [110]. Additionally, there exist several placenta-specific imprinted genes that lack promoter DNA methylation; these loci are instead monoallelically marked with repressive H3K27me3, H3K9me2, and H3K9me3 methylation [111-113]. However, there is no evidence yet that such repressive modifications were present at these loci in oocytes and they could equally depend on oocyte-derived DNA methylation at the corresponding imprinting control region.

While the maternal genome is decorated with H3K9me3 and H3K27me3 in GV oocytes, the paternal genome loses these marks during spermatogenesis [114, 115]. The asymmetry in H3K9me3 methylation between the maternal and paternal genomes persists in the first cell divisions of embryonic development, while both pronuclei are marked with H3K27me3 by the end of the 1-cell stage [116]. There are intriguing parallels between the retention of H3K9-methylated histones and protection from active DNA demethylation in the maternal genome (Figure 4), possibly explaining the oocyte-like methylome seen in pre-implantation embryos and early extra-embryonic tissues [84, 110].

Future perspective

Descriptive studies of DNA methylation in the gametes and early embryo have provided many new avenues for research, but the function of this methylation, especially of maternally-derived methylation, is in many cases still unclear. The striking methylome and modes of transcriptional regulation in the mouse oocyte make it unique from epigenetic, cell signalling, and evolutionary

perspectives. Additionally, even in the post-genomics era the paradigm of genomic imprinting has remained an invaluable model of epigenetic regulation, and will likely continue to be so [15].

Although the histone modifications that characterise the maternal and paternal genomes have been studied extensively by immunofluorescence, it remains largely unknown whether or not these modifications are directly inherited by or play a role in the early embryo. With the rapid advances in chromatin profiling of rare cell populations, the precise localisation of histone marks in the mature oocyte and early embryo will likely soon be known. It is likely that the roles of both gamete-derived and newly deposited histones in the zygote will be complex and multifaceted. This is demonstrably the case for DNA methylation, which appears to be much more dynamic within the zygote than was previously appreciated [96]. And though DNA methylation is seemingly less instructive in transgenerational inheritance than initially speculated, the roles of histone modifications and inherited RNA molecules remain exciting potential transmitters of epigenetic information between generations that deserve further investigation.

The mouse is a powerful model for studying DNA methylation establishment *in vivo*. Rapid advances in next-generation sequencing technologies, especially those adapted to single cells or low cell numbers, are revealing the complex interplay between DNA methylation, histone modifications, and other epigenetic factors during gametogenesis and early mouse development. These developments have also initiated the advent of parallel analyses in human oocytes and early embryos, revealing important similarities and differences between mouse and human [76, 117, 118]. These studies will continue to expand our understanding of mammalian development, and will also shed light on how well murine gametogenesis and embryogenesis model these processes in human.

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Executive summary

DNA methylation establishment in gametes

- Starting from essentially unmethylated genomes in PGCs, gametogenesis results in distinctive DNA methylation landscapes in mature oocytes and sperm.
- In oocytes, DNA methylation is overwhelmingly associated with gene bodies, and transcription has been functionally demonstrated to determine methylated domains, including the DMRs of imprinted genes.
- In the male germline, DNMT3B has a specific role in methylation of transposable elements associated with piRNAs, although the underlying mechanism is unresolved.
- It is not understood why the methylomes of oocyte and sperm are strikingly different, since in mouse both depend mainly on DNMT3A and DNMT3L.

Histone modifications and DNA methylation establishment

- Germ cells provide important *in vivo* systems to interrogate the relationship between histone post-translational modifications and *de novo* DNA methylation.
- In oocytes, transcription may lead to recruitment of DNMT3A via transcription-coupled deposition of H3K36me3, analogous to the targeting of DNMT3B observed in ESCs but indicating cell-specific effects.
- In oocytes, transcription is involved in removing H3K4 methylation at imprinted DMRs, which is inhibitory to DNA methylation.
- Deleting the H3K4me2 demethylase KDM1B does not equally affect CGIs destined for methylation in oocytes, suggesting different loci require different combinations of factors to render them susceptible to DNA methylation.

Roles of DNA methylation in sperm and oocytes, and its fate in the embryo

- Spermatogenesis fails in the absence of DNA methylation, but oocytes can develop normally.
- Despite extensive active and passive demethylation in the zygote and cleavage-stage embryo, sites of oocyte-derived methylation outside imprinted genes are retained and are essential for development, particularly of the placenta.
- The contribution of DNA methylation abnormalities to transgenerational epigenetic inheritance remains contentious and requires further investigation.

DNA methylation and histone modifications in the early embryo

- There is extensive asymmetry of histone modifications between the parental genomes at fertilisation and in their dynamics after fertilisation.

- The co-occurrence of repressive histone modifications, such as H3K9me2 and H3K9me3, may be required to protect imprinted DMRs and other sequence elements against demethylation in the zygote and early embryo.
- Sophisticated genetic studies, as well as advances in low-cell ChIP-seq, will be required to elucidate fully the relationships between gamete-derived DNA methylation and histone modifications in the early embryo.

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Figure Legends

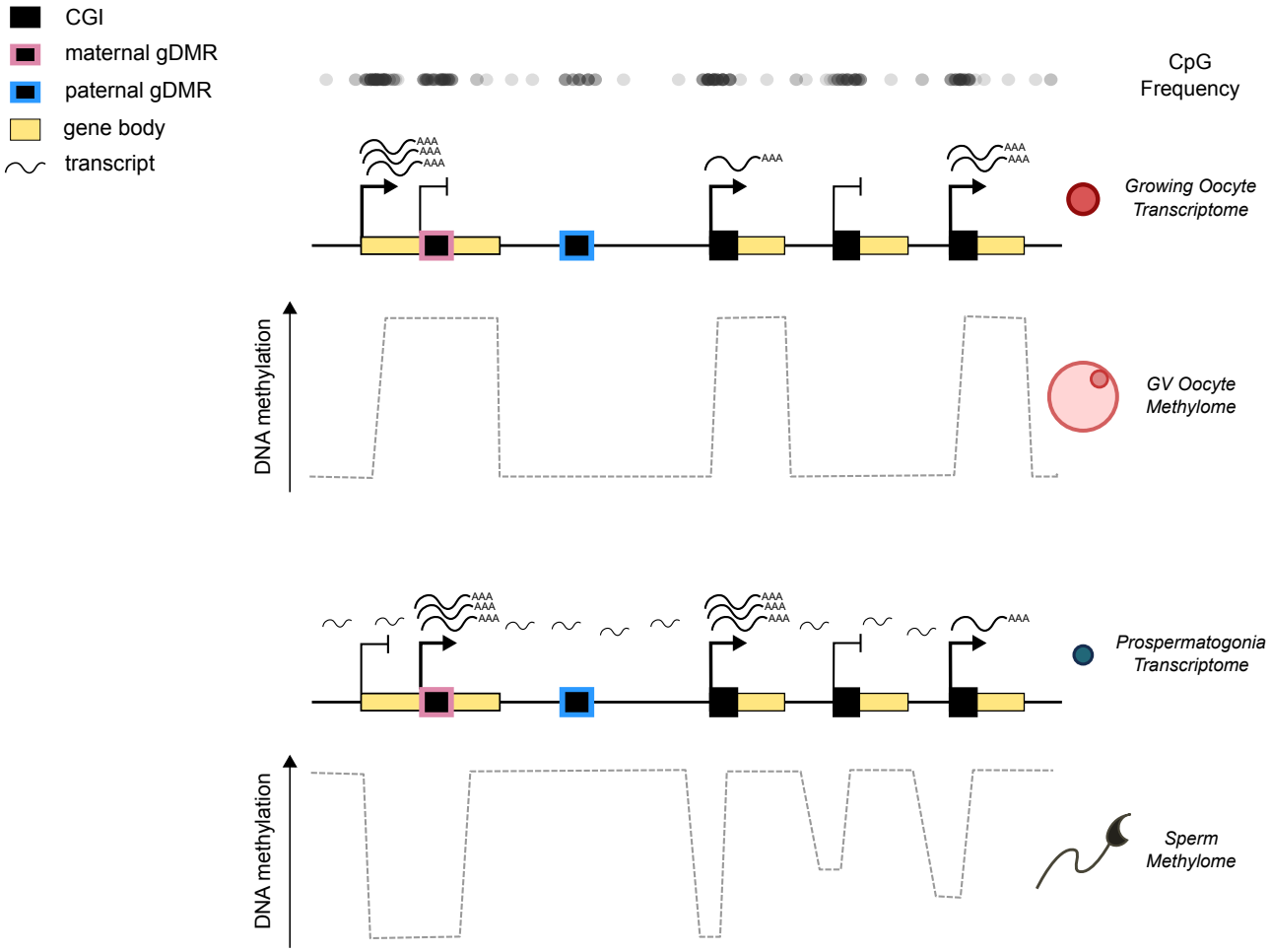
Figure 1. The oocyte and sperm methylomes. Methylated regions in the germinal vesicle (GV) oocyte (top) tightly correspond with active transcription units in growing oocytes, initiating from both CGI and non-CGI transcription start sites. CGIs that become *de novo* methylated in oocytes, including maternally imprinted gDMRs, tend to be within active transcription units and CpG-rich. In the male germline, regions that were both transcribed and untranscribed in prospermatogonia are methylated in mature sperm (bottom). Promoters of genes with weak or no expression in prospermatogonia display intermediate methylation in sperm, from either heterogeneous methylation of these regions in the germ cell pool or methylation heterogeneity in individual CpGs at these genomic regions. Paternally imprinted gDMRs tend to be less CpG-dense than maternally imprinted gDMRs. Notably, in both oocytes and sperm transcription levels past a low threshold result in full methylation of the transcription unit. Arrows, active transcription start sites; crosses, inactive transcription start sites.

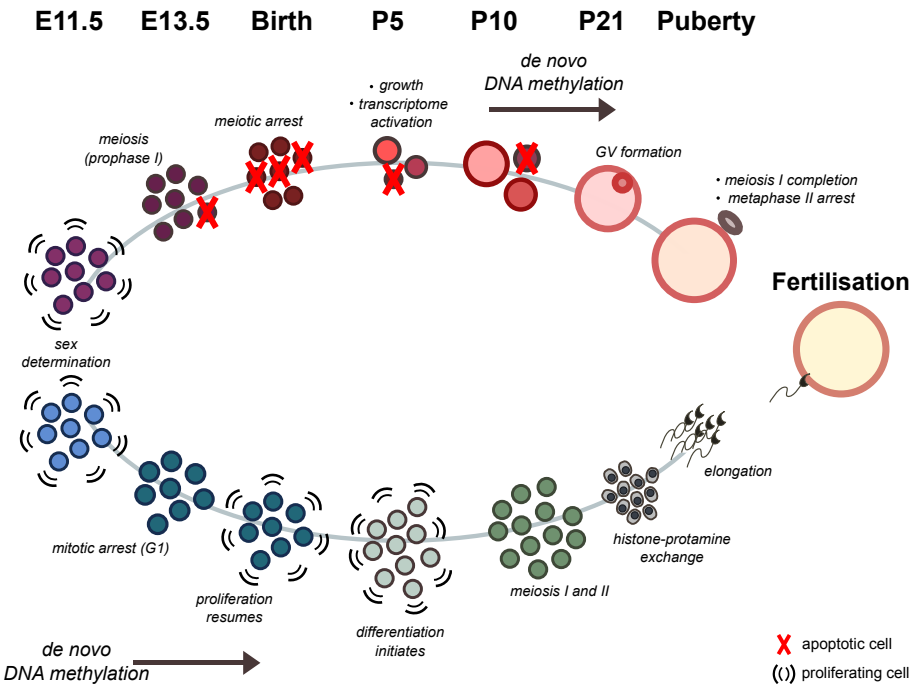
Figure 2. Nuclear and epigenetic dynamics in gametogenesis: temporal and developmental perspectives. In mice, sex determination occurs in the gonadal ridge between E10.5 and E12.5. At E13.5, PGCs in both male and female developing gonads stop proliferating: female PGCs enter meiosis, while male PGCs (called prospermatogonia or gonocytes) arrest in mitotic G1 and undergo *de novo* DNA methylation between E13.5 and birth. By birth (approximately 19 days after conception), female PGCs have become primary oocytes, and arrest at the diplotene stage of meiotic prophase I. Many oocytes undergo apoptosis at this time, and oocytes continue to die throughout oogenesis. Male gametes, now spermatogonia, resume proliferation. At P5, oocytes begin expanding their cytoplasmic volume, and oocyte-specific transcription units also become active. This oocyte growth is asynchronous, with some oocytes growing faster than others. Spermatogonia begin to differentiate. *De novo* methylation begins in oocytes at around P10, or when an oocyte reaches at least 40-45 μm in diameter. Spermatogonia undergo meiosis at this time. Histone-protamine exchange occurs in round spermatids at P21, which later elongate into mature spermatids. At P21, the oocyte methylome is established, and germinal vesicles (GVs) form. Upon ovulation, oocytes become developmentally competent by completing meiosis I and arresting in metaphase II, while extruding the first polar body.

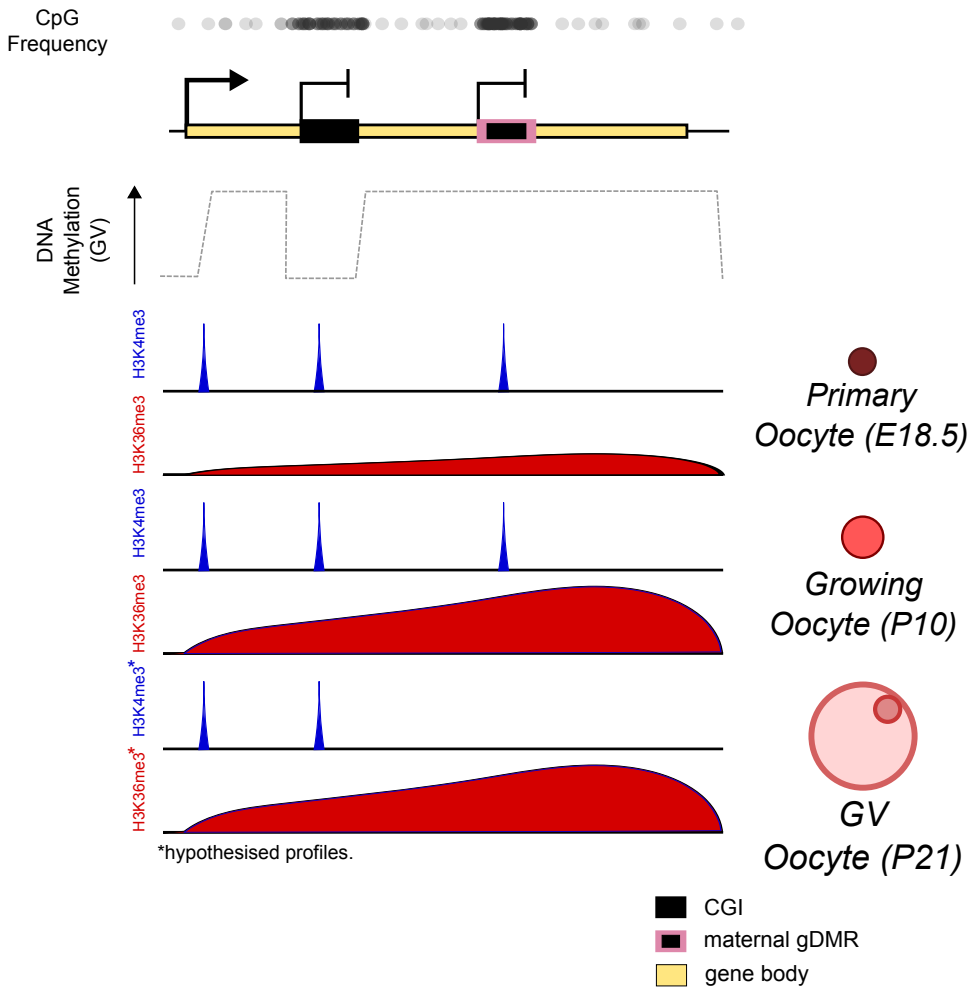
Figure 3. Chromatin modulation accompanies establishment of the oocyte DNA methylome. CGIs and maternally imprinted gDMRs that gain methylation in the oocyte tend to be within active

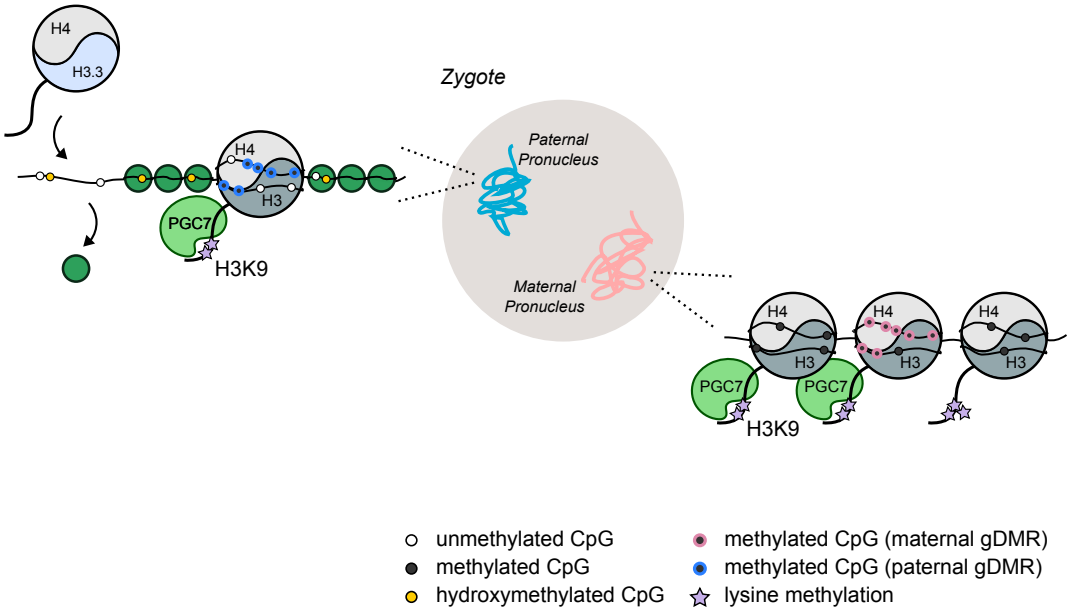
transcription units, but not all transcribed CGIs acquire DNA methylation (top). In the primary (E18.5) oocyte, both CGIs that gain DNA methylation and those that do not are marked with H3K4me3. In growing (P10) oocytes, most CGIs destined for DNA methylation retain this H3K4me3 and, compared with CGIs that resist DNA methylation, they also become enriched in H3K36me3. Given the well-established refractory relationship between H3K4 methylation and DNA methylation, it is reasonable to expect that DNA-methylated CGIs lose H3K4me3 sometime between P10 and P21, to permit DNA methylation establishment at these loci by the GV oocyte stage.

Figure 4. Gametic epigenomes in the zygote. Following fertilisation, most of the paternal genome is actively demethylated, during which time it is characterised by widespread hydroxymethylation. The few canonical nucleosomes present in mature sperm are predicted to persist in the paternal pronucleus, but in the majority of the genome protamines (green circles) are exchanged for maternally-provided histones containing the H3 variant H3.3. The maternal genome is largely not subject to either of these remodelling events. Nucleosomes marked with H3K9me2 recruit PGC7/STELLA, which protects the maternal genome from demethylation. The same mechanism protects DNA methylation over at least two paternally imprinted gDMRs.


CGI
maternal gDMR
paternal gDMR
gene body
transcript
CpG
Frequency
AAA
Growing Oocyte
Transcriptome
DNA methylation
GV Oocyte
Methylome
Prospermatogonia
Transcriptome
DNA methylation
Sperm
Methylome


E11.5
E13.5
Birth
P5
P10
P21
Puberty
de novo
DNA methylation
• growth
• transcriptome
activation
meiotic arrest
meiosis
(prophase I)
GV formation
• meiosis I completion
• metaphase II arrest
Fertilisation
sex
determination
elongation
histone-protamine
exchange
meiosis I and II
mitotic arrest (G1)
proliferation
resumes
differentiation
initiates
de novo
DNA methylation
apoptotic cell
proliferating cell


CpG
Frequency
DNA
Methylation
(GV)
H3K4me3
H3K36me3
H3K4me3
H3K36me3
H3K4me3*
H3K36me3*
*hypothesised profiles.
Primary
Oocyte (E18.5)
Growing
Oocyte (P10)
GV
Oocyte (P21)
CGI
maternal gDMR
gene body


H4
H3.3
Zygote
Paternal
Pronucleus
H4
H3
PGC7
H3K9
Maternal
Pronucleus
H4
H4
H4
H3
H3
H3
PGC7
PGC7
H3K9
unmethylated CpG
methylated CpG
hydroxymethylated CpG
methylated CpG (maternal gDMR)
methylated CpG (paternal gDMR)
lysine methylation